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Duration of protection of rabbits after vaccination with *Bacillus anthracis* recombinant protective antigen vaccine☆

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Abstract

Long-term protection of rabbits that had been vaccinated with two doses of a recombinant protective antigen (rPA) vaccine was examined against an aerosol spore challenge with the Ames isolate of *Bacillus anthracis* at 6 and 12 months. At 6 months after the primary injection, survival was 74.1% (20/27) with quantitative ELISA titer of 22.3 μg of anti-rPA IgG per millilitre and toxin neutralizing antibody (TNA) assay titer of 332. At 12 months after the primary injection, only 37.5% (9/24) of the rabbits were protected with quantitative ELISA titer of 19.8 μg of anti-rPA IgG per millilitre and TNA assay titer of 286. There was a significant loss of protection ($p = 0.0117$) and a significant difference in survival curves ($p = 0.0157$) between the 6- and 12-month groups. When ELISA or TNA assay titer, gender, and challenge dose were entered into a forward logistic regression model, week 26 ELISA titer ($p = 0.0236$) and week 13 TNA assay titer ($p = 0.0147$) for the 6-month group, and week 26 ELISA titer ($p = 0.0326$) and week 8 TNA assay titer ($p = 0.0190$) for the 12-month group, were significant predictors of survival. Neither gender nor challenge dose were identified as having a statistically significant effect on survival. Booster vaccinations with rPA may be required for the long-term protection of rabbits against anthrax.
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Keywords: Anthrax; rPA vaccine; Immunity

1. Introduction

Protection against infection with *Bacillus anthracis* is afforded by a cell-free, FDA-licensed vaccine, Anthrax Vaccine Adsorbed Biothrax (AVA Biothrax; BioPort Corporation, Lansing, MI, USA). AVA Biothrax is prepared by adsorbing filtered culture supernatant fluid from a toxigenic, unencapsulated strain of *B. anthracis*, V770-NP1-R, onto an aluminum hydroxide gel adjuvant. The vaccine contains protective antigen (PA), lethal factor (LF), and various bacterial products which are adsorbed onto the adjuvant [1]. The bipartite anthrax exotoxins, lethal toxin and edema toxin, are formed using the shared constituent PA combined with LF or edema factor (EF), respectively [2]. The major protective component of AVA Biothrax is PA [3–5].

☆ Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Army. The research described herein was sponsored by the US Army Medical Research and Materiel Command, Project 02-4-CC-008.

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14. ABSTRACT

Long-term protection of rabbits that had been vaccinated with two doses of a recombinant protective antigen (rPA) vaccine was examined against an aerosol spore challenge with the Ames isolate of Bacillus anthracis at 6 and 12 months. At 6 months after the primary injection, survival was 74.1% (20/27) with quantitative ELISA titer of 22.3 microg of anti-rPA IgG per millilitre and toxin neutralizing antibody (TNA) assay titer of 332. At 12 months after the primary injection, only 37.5% (9/24) of the rabbits were protected with quantitative ELISA titer of 19.8 microg of anti-rPA IgG per millilitre and TNA assay titer of 286. There was a significant loss of protection (p = 0.0117) and a significant difference in survival curves (p = 0.0157) between the 6- and 12-month groups. When ELISA or TNA assay titer, gender, and challenge dose were entered into a forward logistic regression model, week 26 ELISA titer (p = 0.0236) and week 13 TNA assay titer (p = 0.0147) for the 6-month group, and week 26 ELISA titer (p = 0.0326) and week 8 TNA assay titer (p = 0.0190) for the 12-month group, were significant predictors of survival. Neither gender nor challenge dose were identified as having a statistically significant effect on survival. Booster vaccinations with rPA may be required for the long-term protection of rabbits against anthrax.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, experimental infection, recombinant vaccine, duration of immunity, laboratory animals, rabbits

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In addition to the non-human primate model for anthrax [6,7], the New Zealand white rabbit is considered to be an appropriate animal model for human inhalation anthrax [8]. In studies evaluating the efficacy of AVA and recombinant protective antigen (rPA) vaccines as well as developing in vitro surrogate markers, both the quantitative anti-rPA IgG ELISA and toxin neutralizing antibody (TNA) assay were determined to support serological correlates of immunity in the rabbit aerosol challenge model [9,10]. Long-term protection studies (1–2 years) have been conducted in non-human primates using vaccine preparations similar to AVA [11]. A question that remains unanswered is the long-term efficacy of the rPA vaccine in the rabbit aerosol model. For these studies, rabbits were vaccinated intramuscularly (i.m.) with rPA vaccine preparations at 0 and 4 weeks (primary and secondary vaccinations, respectively) then challenged by the aerosol route either at 6 or 12 months after the primary vaccination. Antibody titers were measured periodically by a quantitative anti-rPA IgG ELISA and a TNA assay, the latter which measures functional antibody activity against lethal toxin cytotoxicity in vitro.

2. Materials and methods

2.1. Animals

An equal number of male and female New Zealand white (NZW) rabbits (3.0–3.5 kg) (Covance Research Products, Denver, PA, USA) were used in the study. The animals received food and water ad libitum. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Vaccination and challenge of rabbits

Recombinant PA (rPA), expressed in a *B. anthracis* background [5,12] was manufactured as a cGMP lot by the Biopharmaceutical Production Facility at NCI-FCRC (Frederick, MD, USA) using a modification of a reported procedure [13]. The same lot of rPA was used throughout these experiments for vaccinations and serological analysis of antibody response. Lethal factor was prepared as previously described [14]. Recombinant PA was adsorbed to Alhydrogel (2% Al_2O_3; HCL Biosector (formerly Superfos Biosector) Frederikssund, Denmark) for 20–24 h at 4 °C before use. NZW rabbits were vaccinated i.m. with 50 μg of rPA vaccine preparations adsorbed to 0.5 mg of aluminum per injection in 0.5 ml volumes at 0 and 4 weeks. There were 28 rabbits (14 male and 14 female) in the 6-month vaccinated group and 24 rabbits (12 male and 12 female) in the 12-month vaccinated group. In the statistical analysis of the data, one female rabbit from the 6-month vaccinated group was identified as an outlier and was removed from the analysis. A group of unvaccinated rabbits (two male and two female) served as challenge controls at each challenge date. At either 6 or 12 months post primary vaccination, rabbits were exposed (head only) to a small-particle aerosol in a modified Henderson exposure system contained within a class III biological safety cabinet with a lethal dose of spores from the Ames isolate of *B. anthracis* [9]. Inhaled doses were calculated using the aerosol exposure concentration obtained from plate counts from the all-glass impinger which continuously sampled the test atmosphere during the 10 min exposure time and the respiratory minute volume for each animal [9]. Spores were prepared as previously described [9] and the same lot of spores was used for both challenge dates. Survival was noted for 21 days after challenge. The time-to death (day) was expressed as the average ± standard deviation (S.D.). The aerosol LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores [9]. The inhaled dose of spores (average LD_{50} ± S.D.) at the 6-month challenge for the vaccinated rabbits was 374 ± 182.0 LD_{50} (4.1×10^7 spores) and for the control rabbits it was 502 ± 98.2 LD_{50} (5.5×10^7 spores). At the 12-month challenge, the inhaled dose of spores (average LD_{50} ± S.D.) was 669 ± 150.6 LD_{50} (7.4×10^7 spores) for the vaccinated rabbits and for the control rabbits it was 650 ± 106.4 LD_{50} (7.2×10^7 spores).

2.3. Serological analysis of antibodies

Blood was collected periodically for analysis of serum antibodies by a quantitative anti-rPA IgG ELISA and TNA assay [10]. ELISA titers were determined by interpolating the average absorbance value for triplicate wells of each sample with the absorbance values of a standard curve generated from seven dilutions of affinity purified rabbit anti-rPA IgG by linear regression analysis and reported as micrograms of anti-rPA IgG per ml (KC4 software, BioTek Instruments, Winooski, VT, USA) [9,10]. Titers were presented as the geometric mean ×/÷ standard error of the geometric mean (S.E.M.). For the TNA assay, the average absorbance value of triplicate wells for each test sample dilution, less the average absorbance value of triplicate wells incubated with lethal toxin, was divided by the average absorbance value of control wells that contained only medium, less the average absorbance value of triplicate wells incubated with lethal toxin, and the ratio multiplied by 100 to obtain the percent viability of the test wells compared to the control wells;

$$\%\,\text{Control} = \frac{\text{sample avg} - \text{lethal toxin avg}}{\text{medium control avg} - \text{lethal toxin avg}} \times 100.$$

The percent control values were plotted against each respective test dilution using a 4-parameter logistic equation algorithm and TNA assay titers were expressed as the reciprocal of the dilution of antiserum that neutralized the cytotoxic

activity of lethal toxin on J774A.1 cells at 50% of control values (ED_{50}) using XLfit software (IDBS, Inc., Emeryville, CA, USA). Titers were presented as the geometric mean ×/÷ S.E.M.

2.4. Data analysis

Log_{10} transformations were applied to all ELISA and TNA assay titers. After transformation, the dependent variable met assumptions of normality and homogeneity of variance. Titers from one female rabbit in the 6-month group were found to be outliers [15] and were excluded from the statistical analysis. Pearson correlation coefficients were calculated between ELISA and TNA assay titers. Mixed model analysis of variance (ANOVA) was used to compare titers between gender, over time, and between challenge groups. The effects of gender and ELISA titer or TNA assay titer on the probability of survival were assessed using a backward-selection logistic regression model. The effects of gender, ELISA or TNA assay titer, and challenge dose on the probability of survival were assessed using a forward-selection logistic regression model. Survival analysis was performed using the Kaplan–Meier method, with log rank tests for comparison of survival curves, which is a plot of the percent survival as a function of time. Fisher exact tests and chi-square tests for proportions were used to compare survival rates, which are the ratio between survivors and the total number of test animals at the end of the study. Analyses were conducted using SAS Version 8.2 (SAS Institute, Inc., SAS OnlineDoc, Version 8, Cary, NC, USA).

3. Results and discussion

Before the booster injection at 4 weeks, the ELISA titers were 21.1 μg of anti-rPA IgG per millilitre and 17.6 μg of anti-rPA IgG per ml for the 6- and 12-month groups, respectively, and the TNA assay ED_{50} titers were 86.4 and 63.6 for the 6- and 12-month groups, respectively (Table 1). Peak serological titers were reached at week 6, which was 2 weeks after the booster injection with 50 μg of rPA given at week 4. At week 6, the ELISA titers were 384.2 μg of anti-rPA IgG per millilitre and 294.8 μg of anti-rPA IgG per millilitre for the 6- and 12-month groups, respectively, and the TNA assay ED_{50} titers were 4641 and 3335 for the 6- and 12-month groups, respectively (Table 1). TNA assay ED_{50} titers were significantly different between the 6- and 12-month groups at week 6 ($p = 0.0068$) and week 8 ($p = 0.0046$). We cannot explain the reason for the significant differences in the TNA assay ED_{50} titers between the 6-month and 12-month groups at weeks 6 and 8. The same lot of rPA was used to prepare the vaccine for either the primary or secondary vaccination of the rabbits. After week 6, antibody responses gradually declined to week 26 (6 months) and then generally remained at that concentration until week 52 for the 12-month group with an ELISA titer of 19.8 μg of anti-rPA IgG per millilitre and a TNA assay ED_{50} titer of 286 (Table 1). While the ELISA titers at weeks 26, 39, and 52 were similar with those measured at week 4, the TNA assay titers were about four-fold higher at weeks 26, 39, and 52 than those measured at week 4 prior to the booster injection. Two weeks after the booster vaccination, the TNA assay ED_{50} titers increased by about 53-fold compared to about a 18-fold increase in the anti-rPA IgG ELISA titers. The fold-increase difference between the TNA assay and ELISA titers might suggest differences between the presentation of the epitopes of rPA available in solution (native conformation) and bound to plastic (denatured epitopes) or it might be the effect of the adjuvant on the antibody response to antigenic determinants on rPA.

Aerosol challenge of the vaccinated rabbits at 6 months (week 26) with 374 ± 182.0 LD_{50} spores of the Ames isolate of *B. anthracis* resulted in 74.1% survival (20/27) with an average time-to-death of 4.0 ± 0.82 days. Challenge control rabbits ($n = 4$) received 502 ± 98.2 LD_{50} Ames spores and had an average time-to-death of 2.3 ± 0.5 days. ELISA and TNA assay ED_{50} titers from the 6-month group that survived

Table 1
Quantitative anti-rPA IgG ELISA and TNA assay titers[a]

Assay	Group	Time post primary injection (week)							
		0	4	6	8	13	26	39	52
ELISA	6-month	BLQ[b]	21.1[c] (1.221)[d]	384.2 (1.086)	220.9 (1.075)	65.2 (1.098)	22.3 (1.142)	na[e]	na
	12-month	BLQ	17.6 (1.380)	294.8 (1.168)	187.7 (1.163)	58.1 (1.134)	24.5 (1.145)	15.3 (1.190)	19.8 (1.257)
TNA	6-month	1.0[f] (na)	86.4 (1.239)	4641[g] (1.058)	2202[h] (1.068)	779 (1.105)	332 (1.127)	na	na
	12-month	1.0 (na)	63.6 (1.436)	3335[g] (1.113)	1551[h] (1.107)	735 (1.127)	307 (1.183)	268 (1.192)	286 (1.215)

[a] Rabbits were inoculated with 50 μg of rPA vaccine at 0 and 4 weeks.
[b] BLQ, below the limit of quantitation which was 0.072 μg/ml IgG, the concentration of the lowest standard (1.44 ng/ml IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.
[c] Micrograms of anti-rPA IgG per millilitre.
[d] Number in parentheses is the S.E.M.
[e] na, not applicable.
[f] The reciprocal of the dilution of serum that protected half of the cells from lethal toxin cytotoxicity (ED_{50}). If the ED_{50} titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of 1.0. The starting dilution for the TNA assay was 1:50.
[g] Titer significantly different between 6- and 12-month group ($p = 0.0068$).
[h] Titer significantly different between 6- and 12-month group ($p = 0.0046$).

Table 2
Quantitative anti-rPA IgG ELISA and TNA assay ED_{50} titers of rabbits that survived or succumbed to aerosol challenge with *B. anthracis* Ames spores[a]

Week	6-month group				12-month group			
	ELISA[b]		TNA assay[c]		ELISA		TNA assay	
	Survivors	Non-survivors	Survivors	Non-survivors	Survivors	Non-survivors	Survivors	Non-survivors
4	20.7[d] (1.304)	22.4[d] (1.197)	80.2[e] (1.335)	107.0[e] (1.034)	19.6[f] (1.688)	16.5[f] (1.525)	82.0[g] (1.877)	54.6[g] (1.570)
6	413.4[d] (1.105)	311.6[d] (1.126)	4932[e] (1.060)	3902[e] (1.076)	362.0[f] (1.262)	260.6[f] (1.228)	4894[g] (1.158)	2650[g] (1.119)
8	227.3[d] (1.088)	203.6[d] (1.157)	2443[e] (1.062)	1636[e] (1.085)	270.2[f] (1.230)	150.9[f] (1.210)	2242[g] (1.135)	1244[g] (1.117)
13	71.8[d] (1.113)	49.6[d] (1.173)	927[e] (1.094)	475[e] (1.123)	77.0[f] (1.212)	49.1[f] (1.166)	1105[g] (1.1569)	576[g] (1.147)
26	27.1[d] (1.151)	12.7[d] (1.242)	394[e] (1.132)	204[e] (1.138)	40.7[f] (1.180)	18.1[f] (1.157)	542[g] (1.192)	218[g] (1.226)
39	na[h]	na	na	na	26.9[f] (1.208)	10.9[f] (1.238)	505[g] (1.200)	183[g] (1.229)
52	na	na	na	na	37.6[f] (1.497)	14.1[f] (1.275)	532[g] (1.467)	205[g] (1.201)

[a] Rabbits were inoculated with 50 μg of rPA vaccine at 0 and 4 weeks.
[b] Micrograms of anti-rPA IgG per millilitre. Number in parentheses is the S.E.M.
[c] The reciprocal of the dilution of serum that protected half of the cells from lethal toxin cytotoxicity (ED_{50}). Number in parentheses is the S.E.M.
[d] Significant differences in quantitative anti-rPA ELISA titers between survivors and non-survivors for the 6-month group at week 26 ($p = 0.0100$), but not at week 4 ($p = 0.8692$), week 6 ($p = 0.1372$), week 8 ($p = 0.5146$), or week 13 ($p = 0.0811$).
[e] Significant differences in TNA assay ED_{50} titers between survivors and non-survivors for the 6-month group at week 8 ($p = 0.0048$), week 13 ($p = 0.0017$), and week 26 ($p = 0.0128$), but not at week 4 ($p = 0.5663$) or week 6 ($p = 0.0655$).
[f] Significant differences in quantitative anti-rPA ELISA titers between survivors and non-survivors for the 12-month group at week 26 ($p = 0.0017$), week 39 ($p = 0.0089$), and week 52 ($p = 0.0378$), but not at week 4 ($p = 0.8035$), week 6 ($p = 0.3173$), week 8 ($p = 0.0602$), or week 13 ($p = 0.0823$).
[g] Significant differences in TNA assay ED_{50} titers between survivors and non-survivors for the 12-month group at week 6 ($p = 0.0030$), week 8 ($p = 0.0025$), or week 13 ($p = 0.0052$), week 26 ($p = 0.0058$), week 39 ($p = 0.0028$), and week 52 ($p = 0.0182$), but not at week 4 ($p = 0.5975$).
[h] na, not applicable.

challenge are compared with those that died from the challenge in Table 2. Except for week 4 serological responses, both the quantitative anti-rPA IgG ELISA and TNA assay ED_{50} titers were higher for rabbits that survived challenge than for rabbits that died from the challenge. Significant differences were measured between survivors and non-survivors quantitative anti-rPA IgG ELISA titer at week 26 ($p = 0.0100$) and TNA assay ED_{50} titers at week 8 ($p = 0.0048$), week 13 ($p = 0.0017$), and week 26 ($p = 0.0128$). There was a significant correlation between ELISA titers and TNA assay ED_{50} titers at week 4, 13, and 26 ($p < 0.0001$) and week 8 ($p = 0.0031$), but not at week 6 ($p = 0.4021$). Significant differences were not measured between genders in survival rates ($p = 0.3845$) (Table 3) nor in survival curves ($p = 0.3155$) for the 6-month challenge group. The mean survival time for males was 15 ± 2.38 days and for females it was 18 ± 2.55 days. Significant differences in ELISA titers between male and female rabbits were observed at week 4 ($p = 0.0075$), week 6 ($p = 0.0232$), week 13 ($p = 0.0057$), and week 26 ($p < 0.0001$), but not at week 8 ($p = 0.2704$) (Table 4). Female rabbits had significantly higher TNA assay ED_{50} titers than male rabbits at week 4 ($p = 0.0235$), week 8 ($p = 0.0350$), week 13 ($p = 0.0003$), and week 26 ($p = 0.0061$), but not at week 6 ($p = 0.3178$) (Table 4). When gender and ELISA titer or TNA assay ED_{50} titer were entered into a backward logistic regression model, the week 26 ELISA titer ($p = 0.0236$) and week 13 TNA assay ED_{50} titer ($p = 0.0147$) were significant predictors of survival.

Table 3
Survival of female and male rabbits inoculated with rPA and challenged by the aerosol route with spores from the Ames strain of *B. anthracis*

Group[a]	Gender	Survivors/total (%)
6-month[b]	Female	11/13 (84.6)
	Male	9/14 (64.3)
12-month[c]	Female	7/12 (58.3)
	Male	2/12 (16.7)

[a] Significant difference in percent survival between the 6- and 12-month groups ($p = 0.0117$) and in survival curves ($p = 0.0157$) were measured.
[b] Mean survival time and standard error for females was 18.23 ± 2.55 days and for males it was 15.07 ± 2.38 days. No significant differences in survival rates between genders was measured ($p = 0.3845$). No significant difference in survival curves between genders was measured ($p = 0.3155$).
[c] Mean survival time and standard error for females was 14.08 ± 2.65 days and for males it was 7.17 ± 1.90 days. No significant differences in survival rates between genders was measured ($p = 0.0894$). However, significant differences in survival curves between genders was determined by Kaplan–Meier log-rank test ($\chi^2(1) = 4.16$, $p = 0.0415$).

Aerosol challenge of rabbits at 12 months (week 52) with 669 ± 150.6 LD_{50} spores of the Ames isolate of *B. anthracis* resulted in 37.5% survival (9/24) with an average time-to-death of 4.4 ± 0.91 days. Challenge control rabbits ($n = 4$) received 650 ± 106.4 LD_{50} spores and had an average time-to-death of 2.8 ± 0.5 days. ELISA and TNA assay titers of the rabbits from the 12-month challenge group that survived challenge are compared with those that died from the challenge in Table 2. Both the quantitative anti-rPA IgG ELISA and TNA assay ED_{50} titers were higher for rabbits that survived challenge than for rabbits that succumbed to the challenge. Significant differences were measured between survivors and non-survivors quantitative anti-rPA IgG ELISA titers at week 26 ($p = 0.0017$), week 39 ($p = 0.0089$), and week 52 ($p = 0.0378$) and TNA assay ED_{50} titers at week 6 ($p = 0.0030$), week 8 ($p = 0.0025$), week 13 ($p = 0.0052$), week 26 ($p = 0.0058$), week 39 ($p = 0.0028$), and week 52 ($p = 0.0182$). There was a significant correlation between ELISA titers and TNA assay ED_{50} titers

at all weeks ($p < 0.0001$). Although there were no significant differences in survival rates between male and female rabbits ($p = 0.0894$), there was a significant difference in survival curves between the genders ($\chi^2(1) = 4.16$, $p = 0.0415$) (Table 3). The mean survival time of female rabbits was 14 ± 2.65 days, whereas the mean survival time for males was 7 ± 1.90 days. Significant differences in ELISA titers between male and female rabbits were observed at week 26 ($p = 0.0203$), week 39 ($p < 0.0001$), and week 52 ($p = 0.0013$) and TNA assay ED_{50} titers between male and female rabbits at week 39 ($p = 0.0003$) and week 52 ($p = 0.0136$) (Table 4). Week 26 ELISA titer ($p = 0.0326$) and week 39 TNA assay ED_{50} titer ($p = 0.0209$) were identified as significant predictors of survival when gender and titer were entered into a backward logistic regression analysis for the 12-month challenge group.

There was a significant loss of protection ($p = 0.0117$) and a significant difference in survival curves ($p = 0.0157$) between the 6- and 12-month groups. A comparison between the quantitative anti-rPA IgG ELISA titers showed no statistical differences in ELISA titers for each time period tested between the two groups. However, as stated above, statistically significant differences were measured between the 6- and 12-month group TNA assay ED_{50} titers at week 6 ($p = 0.0068$) and week 8 ($p = 0.0046$) (Table 1). Differences in survival between the vaccinated rabbits from the 6- and 12-month groups might be attributed to the significantly different aerosol challenge dose ($p < 0.0001$) at 6 and 12 months. The mean and S.D. challenge dose for the 6-month group vaccinated rabbits was 374 ± 182.0 LD_{50} and the mean and SD challenge dose for the 12-month group vaccinated rabbits was 669 ± 150.6 LD_{50}. There was not a significant difference in the challenge dose between 6- and 12-month challenge control groups ($p < 0.0865$). The mean and S.D. challenge dose for the 6-month group challenge control rabbits was 502 ± 98.2 LD_{50} and the mean and S.D. challenge dose for the 12-month group challenge control rabbits was 650 ± 106.4 LD_{50}. The average time-to-death, however, of the vaccinated animals or of the challenge controls were not significantly different between the 6- and 12-month groups. The average time-to-death of the vaccinated rabbits from the 6-month group (4.0 ± 0.82 days) was similar with the average time-to-death of vaccinated rabbits from the 12-month group (4.4 ± 0.91 days). Likewise, the average time-to-death of the challenge control rabbits from the 6-month group (2.3 ± 0.5 days) and the 12-month group (2.8 ± 0.5 days) were similar. Differences between the challenge doses that the animals received at the 6- and 12-month timeframes may be attributed to the greater respiratory capacity that was measured in the older animals in the 12-month group. The calculated challenge dose would be affected by the respiratory minute volume estimates which were derived from direct measurement of respiratory function measurements before exposure. The respiratory minute volume was 1.5-times greater for the 12-month vaccine group (1500 ± 427.9 ml/min) than for the 6-month vaccine group (1037 ± 297.5 ml/min) at the time of challenge. The respiratory minute volume measurements of the four control rabbits from the 12-month group (1467 ± 408.1 ml/min) was only slightly greater than the respiratory minute volume measurements of the four control rabbits from the 6-month group (1229 ± 301.1 ml/min). All the rabbits were placed on the project at same time and were not staggered to adjust for age differences.

As stated above, ELISA titer at week 26 was a predictor of survival for both the 6- and 12-month groups. Significant differences in ELISA titer between survivors and non-survivors (Table 2) and female and male rabbits (Table 4) at various weeks for both the 6- and 12-month groups were also measured. TNA assay ED_{50} titers were also identified as predictors of survival; week 13 for the 6-month group and week 39 for the 12-month group. Significant differences in TNA assay titers between survivors and non-survivors (Table 2) and genders (Table 4) at various weeks were also measured for both the 6- and 12-month groups. Gender differences in survival rates were not observed for the 6- and 12-month groups but gender differences in survival curves were observed only for the 12-month group. At the 12-month timeframe, male rabbits had decreased survival and lower serological responses than female rabbits as measured by ELISA and TNA assay ED_{50} titers. In a previous study [10], we reported that gender had no influence on survival in rabbits vaccinated with rPA vaccine and challenged 4 weeks later. The difference in survival between the 6- and 12-month groups probably was not influenced by the significant difference between the challenge doses. When gender, ELISA titer, and challenge dose were combined within each group, forward logistic regression analysis showed that for both the 6- and 12-month groups, week 26 ELISA titers ($p = 0.0236$ and 0.0326, respectively) again were significant predictors of survival. Similarly, when gender, TNA assay titer, and challenge dose were combined within the 6-month group, week 13 ($p = 0.0147$) remained as a significant predictor of survival in the forward logistic regression model. However, for the 12-month group, week 8 ($p = 0.0190$) remained in the forward logistic regression model as significant predictors of survival instead of week 39 that was identified by the backward logistic regression analysis. The difference between the two results, week 39 or week 8, is attributed to the backward and forward regression analysis model effect. When tested by logistic regression, challenge dose did not have a statistically significant effect on survival outcome ($p = 0.3427$). When gender, titers from week 4 through week 26, and challenge dose were combined for both the 6- and 12-month groups, challenge dose again was not a significant predictor of survival ($p = 0.2281$), while group remained in the equation ($p = 0.0124$).

Early anthrax vaccines were prepared by adsorbing filtered culture supernatant fluids to aluminum potassium sulfate (alum) [11,16,17] or aluminum hydroxide gel [18]. These vaccines provided excellent short-term protection of rabbits and non-human primates against challenge [11,16–18]. Wright et al. [17] observed complete protection of non-

Table 4
Change in quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers between female and male rabbits in the 6- and 12-month challenge groups

Time post initial injection (week)	ELISA titer (μg anti rPA IgG per millilitre)[a]				TNA assay ED_{50} titer[b]			
	6-month group		12-month group		6-month group		12-month group	
	Male	Female	Male	Female	Male	Female	Male	Female
4	12.9[c] (1.27)	30.6[c] (1.30)	28.0[d] (1.28)	11.0[d] (1.78)	54.7[e] (1.40)	141.6[e] (1.20)	103.4[f] (1.25)	39.1[f] (1.96)
6	321.8[c] (1.12)	465.0[c] (1.11)	324.5[d] (1.26)	267.8[d] (1.24)	4903[e] (1.08)	4374[e] (1.09)	2957[f] (1.17)	3763[f] (1.15)
8	204.4[c] (1.10)	240.2[c] (1.11)	183.2[d] (1.22)	192.4[d] (1.27)	1931[e] (1.09)	2536[e] (1.09)	1387[f] (1.15)	1735[f] (1.16)
13	51.5[c] (1.13)	84.3[c] (1.11)	60.0[d] (1.17)	56.2[d] (1.23)	568.3[e] (1.14)	1095[e] (1.08)	677.5[f] (1.16)	798.3[f] (1.21)
26	14.2[c] (1.19)	36.0[c] (1.09)	18.0[d] (1.18)	33.3[d] (1.20)	245.5[e] (1.18)	460.4[e] (1.13)	230.7[f] (1.26)	408.1[f] (1.25)
39	na[g]	na	8.0[d] (1.18)	29.4[d] (1.17)	na	na	150.9[f] (1.21)	475.0[f] (1.21)
52	na	na	10.4[d] (1.24)	40.1[d] (1.35)	na	na	181.6[f] (1.24)	467.9[f] (1.32)

[a] ELISA titer expressed as micrograms of anti-rPA IgG per millilitre. Number in parentheses is the S.E.M.
[b] TNA assay titer expressed as the reciprocal of the dilution of serum that protected half of the cells from lethal toxin cytotoxicity (ED_{50}). Number in parentheses is the S.E.M.
[c] Significant differences in quantitative anti-rPA IgG ELISA titers between genders for week 4 ($p = 0.0075$), week 6 ($p = 0.0232$), week 13 ($p = 0.0057$) and week 26 ($p = <0.0001$) but not for week 8 ($p = 0.2704$).
[d] Significant differences in quantitative anti-rPA IgG ELISA titers between genders for week 26 ($p = 0.0203$), week 39 ($p = <0.0001$), and week 52 ($p = 0.0013$) but not for week 4 ($p = 0.1527$), week 6 ($p = 0.5492$), week 8 ($p = 0.8752$), or week 13 ($p = 0.7996$).
[e] Significant differences in TNA assay ED_{50} titers between genders for week 4 ($p = 0.0235$), week 8 ($p = 0.0350$), week 13 ($p = 0.0003$), and week 52 ($p = 0.0061$) but not for week 6 ($p = 0.3178$).
[f] Significant differences in TNA assay ED_{50} titers between genders for week 39 ($p = 0.0003$) and week 52 ($p = 0.0136$) but not for week 4 ($p = 0.1850$), week 6 ($p = 0.2691$), week 8 ($p = 0.2781$), week 13 ($p = 0.5035$), or week 26 ($p = 0.0898$).
[g] na, not applicable.

human primates with two doses of alum-precipitated vaccine injected at 0 and 2 weeks against an intracutaneous challenge with *B. anthracis* Vollum spores (between 50,000 and 100,000 spores) after 1 year and against a Vollum aerosol spore challenge (8.9×10^5 to 3×10^6 spores) after 34 days. Darlow et al. [11] also protected non-human primates with two doses of an alum precipitated vaccine inoculated at 10-day intervals against an aerosol challenge with approximately 10–15 LD_{50} of *B. anthracis* M.36 after 1 year (100%, 10/10) and after 2 years (85%, 6/7). Ivins et al. [19] reported that non-human primates were fully protected against lethal aerosol challenge with Ames spores 6 weeks after vaccination with a single dose of AVA (100%, 10/10) or with 50 μg of rPA vaccine (100%, 10/10). The antibody titer of non-human primates inoculated with anthrax vaccine preparations has been reported to decrease over time. Darlow et al. [11] observed a steady decrease in the antibody titer in non-human primates during a 2-year period. At 2 years, no detectable antibody titer was measured. Ivins et al. [19] reported that non-human primates inoculated with a single dose of PA adsorbed to alhydrogel or AVA had a decreasing anti-PA IgG ELISA titer after the peak titer at week 5.

As mentioned above, rabbits have been reported to be protected against a parenteral or aerosol challenge in short-term efficacy studies with early anthrax vaccines [16,17] or AVA [9]. In the absence of human clinical trials, which cannot be ethically conducted, or field trials, which are not possible, the US Food and Drug Agency has published regulations to allow appropriate animal efficacy studies for vaccines against anthrax. In an effort to meet this regulation, rabbits have been developed as a surrogate animal model in addition to the non-human primate [20]. Short-term efficacy studies using rabbits have shown that a single dose of rPA vaccine (100 μg) protected 93.3% of rabbits (28/30) at 4 weeks and that with two doses of 10 μg of rPA vaccine at 0 and 4 weeks survival was 100% (12/12) at 6 weeks after the second injection [10]. Unlike non-human primates, however, the duration of protection against infection of rabbits does not appear to be long-term. Wright et al. [17] inoculated rabbits with five doses of an alum-precipitated vaccine preparation on alternate days and observed 50% protection at 16 weeks and only a delay in time to death at 23 weeks against a parenteral challenge with *B. anthracis* Vollum spores. Boor [21] vaccinated rabbits with three 1 ml doses of a cell-free antigen preparation (prepared without an adjuvant) at 6-day intervals and observed a gradual decrease in protection against an intradermal challenge with spores of the CD25 (also referenced as M.36) strain of *B. anthracis* from weeks 1 to 8 and no protection at week 10. Challenge times were relative to the third vaccine dose. In the current study, we observed limited protection of rabbits 6 months (74.1%) and 1 year (37.5%) after injection of two doses of 50 μg of rPA adsorbed to alhydrogel against an aerosol challenge with *B. anthracis* Ames spores. In our previous study, after the peak antibody titer was measured at 2 weeks after a single dose of rPA vaccine, we observed a steady decrease in the ELISA antibody titer as well as with the TNA assay ED_{50} titer [10]. Similarly, after two doses of 10 μg of rPA vaccine, peak ELISA titers of 416 μg anti-rPA IgG and TNA assay ED_{50} titer of 4270 were measured at week 6, after which a decrease in ELISA antibody titer and TNA assay ED_{50} titer were measured at week 10 [10]. In the present study, we also observed a steady decrease in antibody titers from week 6 until the 6 month time point, after which the measured titers remained relatively unchanged until the 12-month time frame, which was the end of this study. The titers that were measured at 6 and 12 months, however, were not simi-

lar in that they were not indicative of comparable protective capacity against a lethal spore challenge. One explanation for the difference in long-term protection between the two animal models may be related to the greater susceptibility of rabbits to infection [8]. The more rapid development of anthrax in the rabbit, compared to the non-human primate [8], may not allow for an adequate amount of time for immunological memory to mount an effective protective response against the infection. Also, the interrelationship between PA-specific memory B cells (humoral immunity) and T cells (cellular immunity) against inhalation anthrax has yet to be determined in the animal models. The role of humoral and cell-mediated immunity in the non-human primate is undergoing extensive research [20]. A recent report by Marcus et al. [22] described immunological memory in guinea pigs vaccinated with rPA and challenged intradermally with Vollum. They found that protection was achieved only after protective levels of neutralizing antibodies were measured 8 days after a booster injection [22].

Humans also demonstrate a decreasing serum antibody concentration to PA after vaccination with AVA over time. In a clinical trial, in which the route and dosing schedule of AVA were evaluated, the peak ELISA titer of human subjects inoculated at 0 and 4 weeks occurred at week 6 and were measured at about 550 μg of anti-rPA IgG per millilitre [23]. At 24 weeks, the ELISA titer was approximately 40 μg of anti-PA IgG per millilitre [23]. Darlow et al. [11] argued that the initial two doses of alum-precipitated vaccine given at 0 and 10 days were insufficient to produce an adequate long-term immunological response in humans based upon the non-human primate data and that a yearly booster injection was needed. As already noted above, in spite of an absent immunological response in the non-human primate, full protection against infection was observed. The immunological response, however, resulting from the booster injection also decreased by half within 1 year [11]. The gradual decline in antibody titer over time in rabbits, non-human primates, and humans, which is the parameter that is currently used to determine the immunological status after vaccination, argues for periodic booster inoculations to maintain an appreciable titer. Further studies are necessary in order to understand the immunological responses after vaccination and the role of immunological memory in the rabbit and non-human primate surrogate models.

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